Identification of three *Aulacaspis* species (Hemiptera: Coccoidea: Diaspididae) by rapid molecular diagnostic technique for quarantine application in Taiwan

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ABSTRACT

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Scale insects are small and similar in morphology, and the identification of species especially in the immature stages is difficult. In this study, a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique was developed to identify three ***Aulacaspis*** species, ***A. murrayae***, ***A. yabunikkei*** and ***A. yasumatsui***. Primers ITS5 and ITS4 were used to amplify the internal transcribed spacer (ITS) regions of the ribosomal DNA (rDNA). The genomic DNA isolated from ***A. yasumatsui*** yielded a single fragment after PCR amplification. The fragment size was about 0.6 kb. The other species, ***A. murrayae*** and ***A. yabunikkei***, gave two PCR products. The sizes of the two fragments were about 0.6 kb and 0.67 kb. These three ***Aulacaspis*** species could be easily discriminated according to the species-

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specific patterns by digesting the PCR products with endonucleases *Taq*I, *Msp*I and *Cfo*I.

(Keywords: ***Aulacaspis*** spp., molecular identification, nuclear ribosomal DNA, internal transcribed spacer (ITS), PCR-RFLP)

INTRODUCTION

Scale insects, ***Aulacaspis*** species, are widely distributed throughout the world. They have tiny bodies, strong reproductive ability, and often follow seedlings, seed, fruit, tubers and root tubers to invade new areas, thus causing serious pest problems. However, they are also important both as economic pests in agriculture and international quarantined pests[2,9]. According to survey, cycad aulacaspis scale (***Aulacaspis yasumatsui*** Takagi) has already confirmed to be a new record species in Taiwan, invaded Taiwan causing serious damage to cycads (*Cycas* spp.)[3]. If they disperse or transfer to other host plants, they will cause serious damage to crops. ***Aulacaspis*** is a stenomorphic genus, and identification of the species is difficult even for expert taxonomists. This species can be distinguished only by their female adult morphology because of unstable patterns and inconspicuous characteristics of egg, larvae and male adult[6], posing problems for identification and diagnosis for quarantine.

Currently, there are more than 91 nominal species of ***Aulacaspis*** genus in the world, and 20 nominal species are found in Taiwan according to a survey research[8]. For polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) identification, it is necessary to ensure that RFLP patterns are distinct interspecies. The molecular taxonomy methods identify species according to morphological characteristics. The reliability of molecular identification techniques using ribosomal DNA (rDNA) or mitochondrial DNA (mtDNA) analysis has to be confirmed through morphological background[4]. The goal of this study is to use rDNA/PCR-RFLP molecular diagnostic technique to identify ***A. murrayae*** and ***A. yabunikkei***, which are commonly found in Taiwan, as well as ***A. yasumatsui***, which are suspected to have invaded Taiwan. Molecular diagnostic identification technique and rDNA sequencing analysis are rapid, simple and accurate techniques for identification of quarantine scale pests that can be widely applied to different growing stages of scale and require only a few specimens, either live or dead.

MATERIALS AND METHODS

Survey, collection, rearing and identification

Each scale insect was collected and recorded as experimental material according

to host plants, geographic distributions and seasonal changes in Taiwan. The collected specimens were then taken to the laboratory, and identified according to their morphological characteristics. A specimen database was established for molecular biology technique identification. All other remnant specimens were preserved in 95% ethanol. There are 14 ***Aulacaspis*** genus populations collected and compared in this study (Table 1).

Genomic DNA extraction and polymerase chain reaction (PCR)

Genomic DNA was obtained from a single specimen using the Blood and Tissue Genomic DNA Miniprep System kit (Viogene Inc., Taiwan), following the extraction protocol of the manufacturer. Precipitated DNA was resuspended in 100 μl of double-distilled (dd) H_2O and used to amplify the internal transcribed spacer (ITS) region (ITS1 plus 5.8S and ITS2) sequence

Table 1. Collection information of ***Aulacaspis*** species used in this study

Species	Collect No.	Locality in Taiwan (Prefecture)	Host plant
A. murrayae	1	Daan District, Taipei	Orange jasmine
	2	Bei District, Taichung	Orange jasmine
	3	Sinying District, Tainan	Orange jasmine
	4	Yuli Township, Hualien	Orange jasmine
A. yabunikkei	1	Daan District, Taipei	Camphor tree
	2	Dashi Township, Taoyuan	Camphor tree
	3	Tianwei Township, Changhua	Camphor tree
	4	Taitung City, Taitung	Camphor tree
A. yasumatsui	1	Daan District, Taipei	Cycads
	2	Dashi Township, Taoyuan	Cycads
	3	Jungli City, Taoyuan	Cycads
	4	Tianwei Township, Changhua	Cycads
	5	Fongshan District, Kaohsiung	Cycads
	6	Yanping Township, Taitung	Cycads

of nuclear ribosomal DNA by PCR.

All PCR amplifications were carried out in a GeneAmp PCR System 9700 (Applied Biosystems, Inc., USA). Each PCR was carried out in a final volume of 50 μl with 25 μl of PCR Master Mix 2X (include 0.05 units/μl Taq DNA Polymerase in reaction buffer, 4 mM $MgCl_2$, 0.4 mM dNTP) (MBI Fermentas Inc., EU), 5 μl of each 1.5 μM of primer, and 5 μl of extracted genomic DNA solution. The primers used in this study are listed in Table 2[1,7] and Fig. 1.

Negative controls used ddH_2O in place of scale genomic DNA. The three rDNA regions were amplified using the same reaction mix solution and different PCR protocols. The ITS1 region and ITS2 regions were amplified by using the same PCR conditions. The thermal condition was as follows: an initial denaturation step of 96°C (5 min), then 35 cycles consisting of denaturation at 94°C (30 sec), annealing at 55°C (30 sec), and extension at 72°C (30 sec), followed by a final extension step at 72°C (7 min). The thermal condition of ITS region (ITS1 plus 5.8S and ITS2) was 96°C (5 min), followed by 35 cycles of 94°C (30 sec), 50°C (30 sec), 72°C (1 min) and a final extension step at 72°C (7 min).

RFLP analysis

Fifteen restriction endonucleases (*Taq*I, *Msp*I, *Cfo*I, *Hae*III, *Alu*I, *Sau*3AI, *Rsa*I, *Pst*I, *Sma*I, *Xba*I, *Hin*fI, *Eco*RI, *Hin*dIII, *Bgo*II and *Pvu*II), which had shown to produce digested fragments in three species, were used in this study to digest PCR products. PCR product of 15 μl were incubated in a 5 μl restriction digest solution, containing 5 U endonucleases, 1× manufacturer's buffer and adding ddH_2O up to 20 μl, for 2 hr at 37°C (or 65°C for *Taq*I).

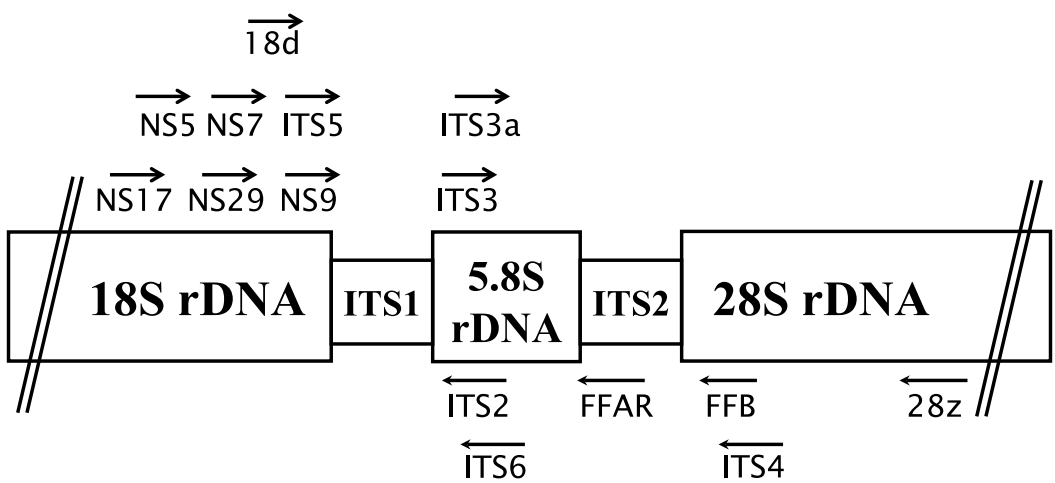

Fig. 1. Locations on nuclear rDNA of PCR primers given in Table 2. The arrowheads represent the 3' end of each primer.

With most enzymes there was no difficulty in digesting the PCR product directly. Restriction fragments were resolved by submerged gel electrophoresis using 8 μl restriction digest solution on a 2% agarose gel containing ethidium bromide and visualized over UV illumination.

RESULTS

PCR amplified from rDNA

This experiment uses three primer sets which are screened from 15 primers to amplify ITS1, ITS2, and 5.8 S of rDNA (Fig. 1; Table 2). The primer sets, ITS5 and ITS2, were used for PCR amplification of ITS1 region. Their effect was better and yielded one fragment following PCR amplification with the sizes of about 290 bp for *A. yabunikkei* and *A. yasumatsui* and 350 bp for *A. murrayae* (Fig. 2). For Amplification of ITS2 region, the primer set, ITS3a and FFB, was better and yielded one fragment following PCR amplification with the size of about 300 bp (data not shown). The combination of primer set, ITS5 and ITS4, could amplify the ITS region of rDNA. Three *Aulacaspis* species yielded one or two fragments following PCR amplification (Fig. 3). The DNA

Table 2. Sequence of PCR primers used in this study

Location	Primer name	Primer sequence (5'→3')
18S of rDNA	NS5	AACTTAAAGGAATTGACGGAAG
	NS7	GAGGCAATAACAGGTCTGTGATGC
	NS9	ACGTGATCACTGTGACGCC
	NS17	TACCAGGTCGAACATAAGTGT
	NS29	GCTTATTGTGTTTGAATGTG
	ITS5	GGAAGTAAAAGTCGTAACAAGG
	18d	CACACCGCCCGTCGCTACTACCGATTG
5.8S of rDNA	ITS3a	TCGATGAAGAACGCAGC
	ITS3	GCATCGATGAAGAACGCAGC
	ITS2	GCTGCGTTCTTCATCGATGC
	ITS6	GAGCCGAGTGATCCACCGCT
28S of rDNA	ITS4	TCCTCCGCTTATTGATATGC
	FFB	TATGCTTAAATTCAGGGGGT
	28z	AGACTCCTTGGTCCGTGTTTCAAGAC
ITS2 of rDNA	FFAR	ATGTGTCCTGCAGTTCACA

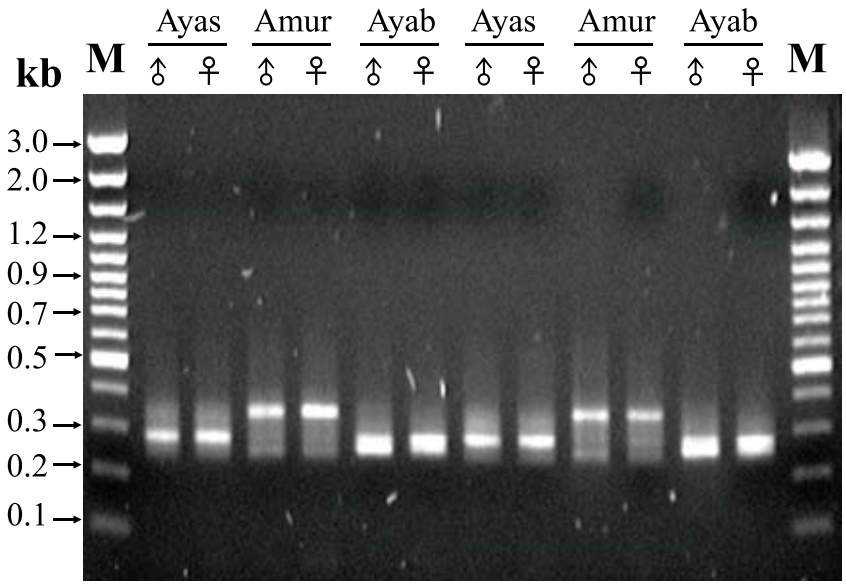

Fig. 2. Amplification pattern of PCR products of the ITS1 regions of the rDNA by using primer set ITS5 and ITS2 on a single individual of the three ***Aulacaspis*** species. ***Aulacaspis*** species: Amur, ***A. murrayae***; Ayab, ***A. yabunikkei***; Ayas, ***A. yasumatsui***. M: GeneRuler™ 100 bp DNA ladder plus maker.

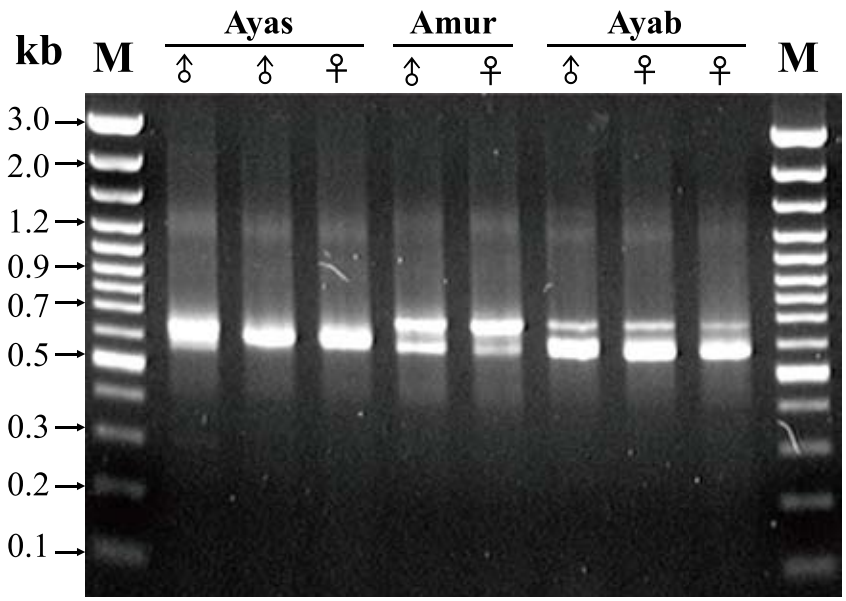

Fig. 3. Amplification patterns of PCR products of the ITS regions of the rDNA by using primer sets ITS5 and ITS4 on a single individual of the three ***Aulacaspis*** species. ***Aulacaspis*** species: Amur, ***A. murrayae***; Ayab, ***A. yabunikkei***; Ayas, ***A. yasumatsui***. M: GeneRuler™ 100 bp DNA ladder plus maker.

isolated from *A. yasumatsui* yielded a single fragment after PCR amplification, with size of about 0.6 kb. The other species *A. murrayae* and *A. yabunikkei* gave two PCR products. The sizes of the two fragments were about 0.6 kb and 0.67 kb, respectively (Fig. 3). Furthermore, there are no difference in DNA amplification fragment among the different stages, sexes, and population of the same scale species.

Restriction endonuclease digestion and RFLP analysis

The primer set, ITS5 and ITS4, was used to amplify the ITS region (ITS1 plus ITS2, 5.8S and small portions of the 18S and 28S) of rDNA. The amplified products were digested with 15 restriction endonucleases, revealing differences in length and numbers of the restriction fragments among the three *Aulacaspis* species. Nine restriction endonucleases (*Bgo*II, *Eco*RI, *Hin*dIII, *Hin*fI, *Pst*I, *Pvu*II, *Rsa*I, *Sma*I and *Xba*I) did not cut the PCR products into digested fragments in some scale species. They were *Pst*I in *A. murrayae*, *Rsa*I , *Hin*fI , *Sma*I and *Pvu*II in *A. yabunikkei* and *A. yasumatsui*, *Xba*I, *Eco*RI, *Hin*dIII and *Bgo*II in *A. murrayae*, *A. yabunikkei* and *A. yasumatsui* (Fig. 4-A; Table 3). *Hae*III produced digested fragments in all three scale species; however, it produced the same restriction fragment patterns in *A. yabunikkei* and *A. yasumatsui* (Fig. 4-B).

Table 3. Species-specific and non-specific DNA fragments obtained after endonuclease digestion of ITS regions of the rDNA from three *Aulacaspis* species

Aulacaspis species [a]			restriction endonuclease
Amur	Ayab	Ayas	
■[b]	■	■	*Taq* Ⅰ , *Msp* Ⅰ , *Cfo* Ⅰ , *Alu* Ⅰ , *Sau*3A Ⅰ
—	□	□	*Pst* Ⅰ
■	—	—	*Rsa* Ⅰ , *Hin*f Ⅰ , *Pvu* Ⅱ , *Sma* Ⅰ
—	—	—	*Xba* Ⅰ , *Eco*R Ⅰ , *Hin*d Ⅲ , *Bgo* Ⅱ
■	□	□	*Hae* Ⅲ

[a] *Aulacaspis* species: Amur, *A. murrayae*; Ayab, *A. yabunikkei*; Ayas, *A. yasumatsui.*

[b] '■': species-specific restriction fragment, '□': non-specific restriction fragment among indicated species, ' —': no restriction fragment.

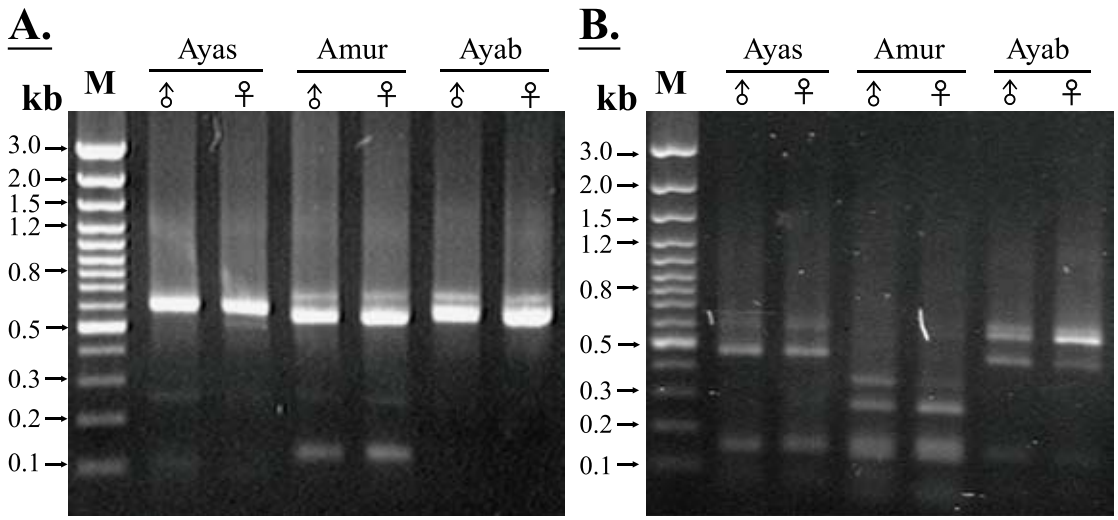

Fig. 4. Inter-species RFLP (endonuclease A. *Rsa* Ⅰ; B. *Hae*Ⅲ) analysis of the ITS region PCR products (primer set ITS5 and ITS4).
Aulacaspis species: Amur, ***A. murrayae***; Ayab, ***A. yabunikkei***; Ayas, ***A. yasumatsui***.
M: GeneRuler™ 100 bp DNA ladder plus maker.

Five of the 15 restriction endonucleases tested (*Tag*I, *Msp*I, *Cfo*I, *Alu*I and *Sau*3AI) cut all of the PCR products and provided species-specific restriction fragment patterns, and thus the three scale species could be easily discriminated from each other (Fig. 5). As for the population examined in this study, no intraspecific variation in the restriction fragment patterns was found among ***A. murrayae***, ***A. yabunikkei*** and ***A. yasumatsui***. The same species is different while collecting to samples of the area, different individuals of the male and female, have the same PCR-RFLP patterns (Fig. 4; Fig. 5). But there were obvious differences between different species. According to the findings, the rDNA (ITS)/PCR-RFLP patterns are recommended for the identification of these ***Aulacaspis*** species.

DISCUSSION

Morphological characteristics commonly used for identification of ***Aulacaspis*** species are shape of prosoma, presence or absence of prosomal tubercle, number of disc pores, shape and width of pygidium, structure and number of pygidial lobes, shape and number of wax glands, site of gonopore, site and shape of anus, number and shape of marginal gland spines, and number of perivular pore clusters[6]. Using these morphological characteristics for ***Aulacaspis*** species identification has the following problems. 1). There are many overlapping morphological diagnostic

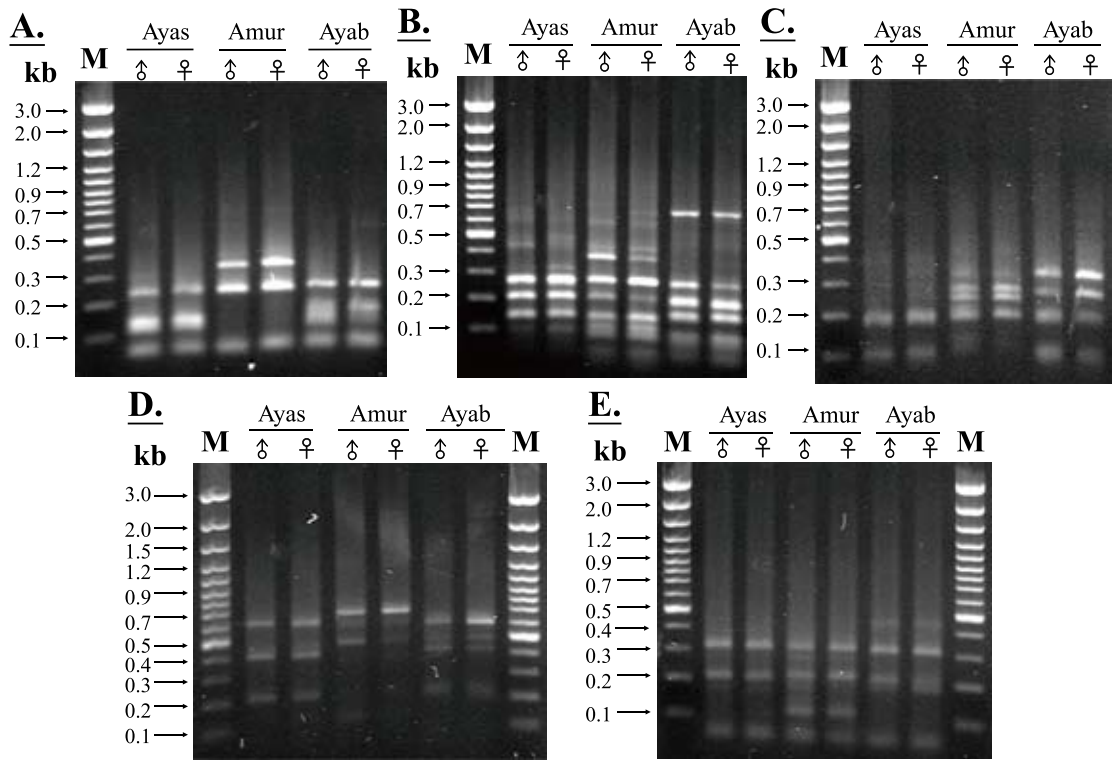

Fig. 5. Inter-species RFLP (endonuclease A. *Taq* I ; B. *Msp* I ; C. *Cfo* I ; D. *Alu* I ; E. *Sau*3A I) analysis of the ITS region PCR products (primer set ITS5 and ITS4). *Aulacaspis* species: Amur, *A. murrayae*; Ayab, *A. yabunikkei*; Ayas, *A. yasumatsui*. M: GeneRuler™ 100 bp DNA ladder plus maker.

characteristics among the species, which make identification difficult even for expert entomologists. 2). *Aulacaspis* species are almost exclusively characterized by adult female morphology. It is nearly impossible to identify them on the morphological basis of adult male or immature stages alone.

In this study, the RFLP patterns of the ITS regions of the rDNA allowed successful discrimination of the three *Aulacaspis* species including *A. murrayae*, *A. yabunikkei* and *A. yasumatsui* on a single scale basis. The restriction patterns have never shown any variations among same *Aulacaspis* species collected from different districts. The RFLP patterns in *Aulacaspis* populations were the same for each species, regardless of the developmental stage and sex. Thus, the rDNA(ITS)/PCR-RFLP method can solve the above-mentioned problems and is a reliable support for the identification of *Aulacaspis* species. The restriction patterns of ITS region with each of *Taq*I, *Msp*I, *Cfo*I, *Alu*I, and *Sau*3AI constantly distinguished the three species examined. It is very likely that RFLP

patterns of the other species of *Aulacaspis* with restriction endonucleases are also species-specific. On the other hand, some other endonucleases, such as *Bgo*II, *Eco*RI, *Hin*dIII, *Hin*fI, *Pst*I, *Pvu*II, *Rsa*I, *Sma*I and *Xba*I, did not digest the amplified products of one, two, or three species in this study (Table 3). Further, the RFLP patterns of HaeIII showed no difference between *A. yabunikkei* and *A. yasumatsui*. Thus, even effective endonucleases, such as *Taq*I, *Msp*I, *Cfo*I, *Alu*I, and *Sau*3AI, may occasionally fail to digest PCR products and to distinguish between restriction patterns in species of unknown RFLP patterns. Therefore, it is important to investigate more than two species-specific RFLP patterns when known or unknown *Aulacaspis* species are distinguished by the rDNA(ITS)/PCR-RFLP method.

When using primer set, ITS5 and ITS4, to amplify ITS region (ITS1 plus ITS2, 5.8S and small portions of the 18S and 28S) of rDNA, the amplification patterns revealed one or two fragments. Two amplification products were also reported in the PCR amplification of rDNA in some nematodes[5]. It is also reported that the PCR products of the ITS regions of some nematodes consistently gave a larger total size of the restriction fragments than that of the PCR product after digestion with some endonucleases. These phenomena suggest that slightly different sequences are present in the two ITS regions within a single individual of the two *Aulacaspis* species. It is presumed that the smeary RFLP patterns observed in *Aulacaspis* species are due to the digestion of two fragments with slightly different sequences. *A. yasumatsui* are suspected to have invaded Taiwan recently, causing serious damage to cycads since 2000. It is difficult to distinguish between *A. murrayae*, *A. yabunikkei* and *A. yasumatsui* by morphological traits, because of their small size and lack of sufficient diagnostic characteristics. Therefore, it remains unknown whether the attack on cycads is due to *A. yasumatsui*. However, these three scale specimens can be distinguished by RFLP patterns.

There are many studies using molecular markers to identify insect species. However, there is no common consensus on which molecular marker is the most representative. The results of this experiment validate the use of molecular diagnostic identification to distinguish three important quarantine *Aulacaspis* species. This molecular technique meets the requirements of international quarantine application. Like other methods, such as morphological characteristics, host plant records, and enzyme electrophoresis, it also could provide accuracy. Our future work would focus on establishing a more complete contrast database-driven expert system and fulfilling rapid molecular diagnostic technique for quarantine scale pest application.

REFERENCES

1. Armstrong, K. F., Cameron, C. M., and Frampton, E. R. 1997. Fruit fly (Diptera: Tephritidae) species identification: a rapid molecular diagnostic technique for quarantine application. Bull. Entomol. Res. 87: 111-118.
2. Burger, H. C., and Ulenberg, S. A. 1990. Chapter 3.2 Quarantine problem and procedures. pp.313-327. *In*: D. Rosen, [ed.], Armored Scale Insect: Their Biology, Natural enemies and Control. Vol. B. Elsevier Science Publishers B. V., Amsterdam, The Netherlands.
3. Chiu, Y. C, Wu, W. J., Lai, P. Y., Shih, H. P., and Shih, C. J. 2003. Study on the ecology and control of cycad scale, ***Aulacaspis yasumatsui*** Takagi (Hemiptera: Diaspididae). pp. 169-191. In:Y. H. Cheng, H. R. Yang, S. H. Huang & H. F. Ni [eds.], Proceedings of the Integrated Management of Crops Pests in Taiwan (TARI Special Publication No. 106). Taiwan Agricultural Research Institute, Taichung, Taiwan. 224 pp.
4. Loxdale, H. D., and Lushai, G. 1998. Molecular markers in entomology. Bull. Entomol. Res. 88: 577-600.
5. Orui, Y., Mizukubo, T. 1999. Discrimination of seven ***Pratylenchus*** species (Nematoda: Pratylenchidae) in Japan by PCR-RFLP analysis. Appl. Entomol. Zool. 34: 205-211.
6. Rosen, D. 1990. Armored Scale Insects: Their Biology, Natural Enemies And Control. Volume A. Elsevier Science Publishers B. V., Amsterdam, The Netherlands.
7. White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. pp. 315-322. *In*: M. A. Innis, D. H. Gelgand, J. J. Sninsky, & T. J. White [eds], PCR Protocols: A Guide to Methods and Applications Academic Press, San Diego.
8. Wu, W. J. 1986. Morphology and taxonomy of the genus Aulacaspis of Taiwan (Hemiptera: Coccoidea: Diaspididae). Doctoral dissertation, Graduate Institute of Plant Pathology and Entomology, National Taiwan Univ., Taipei, Taiwan. 133 pp.
9. Wu, W. J. 1994. Scale insect, pp. 545-678. *In*: Y. H. Tzeng, & C. N. Chen [eds.], Plant Quarantine: Diagnosis of Tiny Animals. Bureau of Standards, Methodology and Inspection. Ministry of Economic Affairs Press, Taiwan. 902 pp.

摘 要

邱一中[1*]、陳淑佩[1]、吳文哲[2,3]、石正人[2]。2013。利用快速分子診斷技術鑑定台灣三種白輪盾介殼蟲（*Aulacaspis* spp.）（半翅目：介殼蟲總科：盾介殼蟲科）。植保會刊 55(4):117-128。([1]行政院農業委員會農業試驗所應用動物組，台中市霧峰區；[2]國立台灣大學昆蟲學系，台北市大安區；[3]國立臺灣大學植物醫學研究中心，台北市大安區)

介殼蟲蟲體微小且外形相似，幼體時期特徵不明顯，在鑑定上不易確定，造成在檢疫工作上困難重重。本研究利用基因組核酸標誌 (genomic DNA markers)，以分子診斷技術鑑別三種白輪盾介殼蟲屬 (*Aulacaspis* spp.) 之介殼蟲，分別為蘇鐵白輪盾介殼蟲 (*A. yasumatsui* Takagi)、月橘白輪盾介殼蟲 (*A. murrayae* Takahashi) 和樟白輪盾介殼蟲 (*A. yabunikkei* Kuwana)。以單隻標本 DNA 進行聚合酶連鎖反應-限制酶片段長度多態型 (polymerase chain reaction-restriction fragment length polymorphism, PCR-RFLP) 的結果分析，利用 PCR 引子對 (ITS5 和 ITS4) 增幅 rDNA 之 ITS 區段，蘇鐵白輪盾介殼蟲增幅出單一片段，大小約 0.6 kb；月橘白輪盾介殼蟲和樟白輪盾介殼蟲則增幅出二片段，大小約 0.6 kb 和 0.67 kb。利用 *Taq*I、*Msp*I 和 *Cfo*I 核酸內切酶進行 ITS/rDNA PCR 產物酶切，所得圖譜可容易區別三種普遍分布且外型進似的白輪盾介殼蟲。

(關鍵詞：白輪盾介殼蟲、分子鑑定、核醣體去氧核醣核酸、轉錄區內空白段、限制性片段長度多態型分析)

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